Construction of a Strain of Bacillus subtilis 168 that Displays the Sublancin Lantibiotic on the Surface of the Cell

This application claims priority under 35 U.S.C. §1.119(e) to provisional application serial no. 60/215,449, filed June 29, 2000.

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Field of the invention

[0001] This invention relates to the construction and expression of a sublancin-derived Lantibody Display Peptide in a strain of Bacillus subtilis and the use thereof.

Background of the Invention

[0002] Lantibodies were initially described by the inventors of this application **(1)**. Lantibodies are derived from lantibiotics which are a family of natural peptides that have antimicrobial activity. Lantibiotics have unique chemical and biological properties that are conferred by the presence of unusual amino acid residues such as dehydroalanine, dehydrobutyrine, lanthionine, and 3-methyllanthionine. The dehydro residues are electrophilic, and are capable of reacting with nucleophilic groups on polypeptide surfaces (1). By constructing suitable polypeptide environments around the dehydro residues, it is possible to control the reactivity and specificity of the dehydro residues, to react in a highly specific way with particular nucleophilic groups on the polypeptide surface. This reaction can alter the biological activity of the polypeptide surface, and if it is on a pathogen such as a bacterium or a virus, the activity of the pathogen can be destroyed. If the polypeptide surface is part of an enzyme, the activity of the enzyme can be altered in some useful way.

[0003] In this disclosure, a novel process by which the lantibodies are designed and constructed is described, and the lantibodies thus produced are versatile in their use. Also disclosed is a novel means for the identification of lantibodies which bind specifically to desired target molecules.

[0004] The inspiration for this invention is the mammalian immune system, in which stem cells differentiate into B-cells. This differentiation involves random recombination events among the variable regions of antibody genes, so that the resulting B-cell becomes programmed for the production of a particular antibody whose antigen-combining regions have been determined by a random process. The antibody that any B-cell can make is then displayed on the surface of the respective B-cell, and this surface antibody can interact with circulating pathogenic antigens. In the event that an antigen binds tightly to one of the displayed antibodies, the binding triggers cell division and further maturation of the B-cell into a plasma cell, which then produces and secretes large quantities of the antibody, which then leads to the destruction of the antigen **(2-3)**.

[0005] Essential features of this natural process include the random generation of a population of antibodies, each of which is produced by a cell that displays the antibody that it is genetically programmed to make. Then, there is a highly-efficient process for the selection and amplification of those antibodies that bind to a specific antigen. The amplification is achieved by stimulating the division of those B-cells that display the antigen-binding antibodies.

[0006] Using discoveries in the Inventor's laboratory, the lantibody that a given bacterial cell produces is displayed on the surface of the cell. It is demonstrated that a population of lantibody producing cells can be exposed to a target molecule, and that the cells whose surface lantibodies can bind to the target can be specifically recovered, so that the population of target-binding cells is enriched. This enriched population can be subjected to repeated selection and enrichment, so that a purified population of the specific antigen-binding cells can be obtained. A very important aspect of this process is that once the target-binding cells are obtained, the lantibody that is displayed on the surface of the cell can be further characterized or the corresponding lantibody peptide can be used to

identify novel nucleophilic groups on target molecules or even novel target molecules.

[0007] Knowledge of the structure of the lantibody is very important, because it can provide the basis of understanding the fundamental principles that are responsible for causing a particular lantibody to bind to a particular antigen. This knowledge can be applied to the rational design of new lantibodies that are directed toward nucleophilic targets, so that the methods by which new lantibodies are made is not solely dependent on random chance.

Summary of the Invention

[0008] An object of the invention is a lantibiotic-spacer-subtilin leader sequence in anchoring a lantibiotic peptide to the cell surface of a host cell. The construction of the lantibiotic-spacer-subtilin chimera comprises lantibiotic structural regions being fused at the C-terminus to a spacer which is fused to the N-terminus of the subtilin signal leader sequence.

[0009] Another object of the invention is a mutagenesis-vector for replacing the endogenous lantibiotic chromosomal gene with a mutagenized lantibiotic sequence in an expression host.

[0010] Another object of the invention is a Bacillus subtilis host strain engineered to contain a deletion of a portion of the *sun A* gene with the remaining portion of the *sunA* gene being flanked by an *erm* gene.

[0011] Another object of the invention is a method for selecting a Lantibody Display Peptide having the ability to bind to a nucleophilic group on a target molecule.

[0012] Another object of the invention is a kit containing a bacterial host cell expressing a Lantibody Display Peptide for use in the identification of a target molecule.

Brief Description of the Figures

[0013] Figure 1. Structure of sublancin 168.

[0014] Figure 2. Structure of sublancin Lantibody Display Peptide.

[0015] Figure 3. Location of template regions for PCR primers used to synthesize fragments used in the construction of mutagenic vectors and host. *B. subtilis* 168 sequence surrounding the sublancin structural gene (*sunA*), which was used to construct mutagenic plasmids is shown. The locations of the template regions of the mutagenic PCR primers are underlined, and the complete sequences of the primers are shown in Table II. Each primer name is accompanied by an arrow that shows the direction of priming. Nucleotide sequences in bold are those that encode the YolF, *SunA*, and N-terminal end of the sunT ORFs, respectively. Gene sequences are from **(4)** and **(5)**.

[0016] Figure 4. Sequence of the EcoRI-HindIII insert of the pLPVc integrative plasmid used to delete and replace the natural *sunA* gene with a mutagenized *sunA* gene in the *B. subtilis* 168 chromosome. A chloramphenicol (*cat*) gene has been inserted at an engineered BamHI site to provide a selective marker. An XhoI site has been engineered into the *sunA* leader region by means of a silent mutation to facilitate the construction of structural mutants. The PstI site at the 3'-end of the *sunA* gene is a natural restriction site. The EcoRI-HindIII fragment is cloned into the EcoRI and HindIII sites of the pTZ *mps* (SEQ ID NO. 1).

[0017] Figure 5. Strategy for the construction of the host-vector pair used to make sublancin mutants. Plasmid pLPH*erm* was linearized and transformed into wild-type *B. subtilis* 168. Double recombinants in which the SunA ORF was replaced by an *erm* gene and the ΔsunA ORF were selected on erythromycin plates. These mutants, which are erythromycin resistant and encode a defective SunA, peptide, were characterized as shown in Figure 5. One of the mutants was selected and designated as *B. subtilis* LPeΔ*sunA*.

[0018] Figure 6. Lack of sublancin production in B. subtilis EΔSun, and its restoration by integration of the *sunA'* gene. Panel A. Halo assay showing sublancin production from wild-type *B. subtilis 168,* compared to the EΔSun deletion strain. Panel B. Halo assay showing production of sublancin after restoration of the sublancin gene as *sunA'*, which has translationally-silent mutations.

[0019] Figure 7. The sequence of *sunA-PG*$_{20}$*-S*$_L$ in pAV2. The reading frame of the *sunA-PG*$_{20}$*-S*$_L$ gene and the sequence of the peptide sequence the gene encodes are highlighted in bold (SEQ ID No. 2). Also highlighted are the restriction sites as well as the leader peptide, sublancin prepeptide, polyglycine, and subtilin leader coding regions of the gene. The sequences flanking the gene correspond to those of the mutagenesis cassette vector pLPcat.

[0020] Figure 8. Lantibody Display Peptide as expressed from *B. subtilis* 168 consists of mature sublancin segment (1-37), a 20-residue polyglycine spacer (38-57) and the subtilin leader segment (58-81) (SEQ ID No. 2).

Detailed Description of the Invention

[0021] The present invention explores the contribution of a spacer-subtilin leader peptide sequence in anchoring a lantibiotic peptide to the cell surface of a host cell. The construction of the lantibiotic-spacer-subtilin chimera comprises lantibiotic structural regions being fused at the C-terminus to a spacer which is fused to the N-terminus of the subtilin signal leader sequence. The inventors have discovered that the chimeras in which the C-terminal portion of the structural region correspond to the spacer-subtilin leader are processed so as to anchor the chimeric molecule to the surface of the host cell, and to give an active lantibiotic product.

[0022] The strategy for displaying the peptide is based on a discovery in the Inventor's laboratory while doing experiments with the lantibiotic called subtilin. A feature of all lantibiotics is that they are expressed as polypeptide precursors that contain a leader

peptide that is cleaved at some stage during the biosynthetic process. In studies to determine the role of the subtilin leader peptide, it was demonstrated that the subtilin leader has a strong affinity for the cell wall of B. subtilis 168, and that a crucial step in subtilin biosynthesis is the proteolytic cleavage of the leader, which results in release of the subtilin into the medium. Without this cleavage, subtilin cannot be released **(7)**.

[0023] This invention is based on the fact that incorporation of the subtilin leader segment into the lantibiotic peptide results in a form of lantibiotic that is retained in the cell wall instead of being released into the medium.

[0024] The term "gene" refers to a polynucleic acid or a nucleotide which encodes a peptide, a prepeptide, a protein or a marker, or to a vector or plasmid containing such a polynucleic acid or nucleotide.

[0025] A "chimera" refers to a fusion peptide or protein which is comprised of a part from a first peptide or protein, and a part from one or more additional proteins or peptides.

[0026] A "mutant" gene or peptide refers to a gene having a sequence in that one or more bases or residues are deleted, substituted or added at any position therein, including either terminus.

[0027] A "Lantibody Display Peptide" refers to a lantibiotic peptide sequence containing a C-terminal amino acid spacer-subtilin leader sequence, which allows for a chimeric peptide or protein product to be expressed on the surface of a host cell through the binding of the chimeric molecule to the cell surface by the subtilin leader peptide. Advantageously, the fusion molecule retains the functional characteristics with respect to the lantibiotic portion of the molecule.

[0028] In the present application, "biological activity" refers to activity against a preferably nucleophilic target molecule. Biological activity includes but is not limited to activity against or for modifying enzymatic activity of an enzyme, inhibiting proliferation or growth of an infectious particle or a cancer cell, or blocking the binding of a ligand to its receptor. Most preferably the activity is against Bacillus cereus spores and/or vegetative cells. Preferably, biological activity against Bacillus cereus spores is measured using the "halo assay" described in the experimental section hereunder.

[0029] The present invention concerns nucleotides, vectors and constructs encoded thereby, which encode a chimeric or mutant lantibiotic polypeptide of the formula:

(lantibiotic)-(spacer)-(subtilin leader peptide)

wherein the lantibiotic is selected from the group consisting of nisin, subtilin, epidermin, pep5, epilancin, duramycin A, duramycin B, duramycin C, cinnamycin, ancovenin, meracidin, actagardine, lacticin 481, streptococcin AFF22, salivaricin A, lactocin S, carnocin IU 49, mutacin II, cytolysin, sublancin, and a mutant of any of the aforementioned lantibiotics. Preferably, the lantibiotic retains its functional characteristics when expressed in a lantibiotic-producing host. More preferably the lantibiotic is sublancin, and most preferably, the sublancin is sublancin 168.

[0030] The present construct includes a peptide spacer comprising from 1 to 40 amino acids, the spacer being of sufficient length and design to produce a region with unstructured secondary conformation. In this regard, non-polar amino acids are preferred. The amino acid is preferably one or more amino acids selected from the group consisting of glycine (G or Gly), alanine (A or Ala), valine (V or Val), isoleucine (I or Ile) and leucine (L or Leu). Preferably the amino acid is glycine.

[0031] The present construct also includes a subtilin leader peptide shown as residues 58-81 of SEQ ID No. 2. The entire sequence is not necessary, however, it has been shown that residues 78-81 are necessary. Therefore, the subtilin leader peptide of the claimed construct can be residues 58-81, 68-81, 73-81 or 78-81 of SEQ ID No. 2.

[0032] "Sublancin 168" (*sun A* gene) is the preferred construction platform for the Lantibody Display Peptide. Sublancin 168 is a lantibiotic that was discovered in the laboratory of the Inventor, the structure of which is shown in Figure 1.

[0033] Sublancin has many attributes that make it an ideal platform on which to construct the Lantibody Display Peptide

[0034] Sublancin is a lantibiotic that is endogenous to the gram-positive bacterium Bacillus subtilis 168, which is a bacterial strain that has been intensely studied. The complete sequence of its genome is known, and excellent tools for genetic manipulation are available. Strain 168 has been widely used for industrial production of genetically-engineered biomaterials, so its use for the industrial production of sublancin 168 derivatives is straightforward. The natural level of sublancin 168 production by strain 168 is good **(4)**, which facilitates efficient production of the derivatives.

[0035] Sublancin 168 is intrinsically highly stable, which enhances the stability of sublancin derivatives.

[0036] Sublancin contains several distinct structural regions, which are defined by the locations of the disulfide bridges. An important aspect is that residues 1-13 have a high propensity for α-helix formation, whereas residues 30-37 have a high propensity for β-sheet formation. In contrast, residues 5-28 are very rich in glycines, which tend to disrupt both α-helix and β-sheet, and possess little, if any secondary structure. Without being bound by scientific theory, it is believed that the 1-13 region of α-helix and the 30-37 region of β-sheet form stable secondary structures, and perhaps tertiary interactions with each other, and constitute a "constant" region, and this constant region is preferably unchanged within the Lantibody Display Peptide. It is the region consisting of residues 15-28, that contain the unusual residues of sublancin, that are preferably subjected to mutagenesis, as this region is conceptually the "variable" region of the lantibodies. It is in this way that the lantibody is conceptually based on the mammalian antibody. It is the variable region of the lantibody that corresponds to the antigen-combining region of the antibody, and the constant regions of the lantibody correspond to the constant, or "framework" regions of the antibody.

[0037] However, there are important fundamental differences between a lantibody and an antibody. Compared to antibodies, which are typically 150,000 Da, lantibodies are very small molecules having molecular weights less than 4,000 Da. This 40-fold difference in

size allows lantibodies to gain ready access to targets that are completely inaccessible by antibodies. Another fundamental difference is the presence of unusual residues in lantibodies, which provide functionalities that antibodies cannot possess. For example, the dehydro residues are electrophilic, and can become covalently attached to specific nucleophilic targets.

[0038] Figure 2 shows how the sublancin prepeptide has been modified to become a Sublancin Display Peptide. It retains the normal sublancin leader sequence at the N-terminal end, which supports the normal functions of posttranslational modifications, translocation through the membrane, and cleaving away in its normal fashion.

[0039] For the peptide to be displayed on the surface of the cell, a spacer has been fused to the C-terminus of the lantiobiotic peptide followed by the subtilin leader sequence. The secondary structure of the chimeric molecule is disrupted by the introduction of the spacer into this region of the molecule, allowing the molecule to extend in an upward direction. The spacer can contain from 1-40 residues, preferably, 10-30 residues, more preferably from 15-25, and most preferably about 20.

[0040] A proviso for the subtilin leader segment is that it retain its affinity for the cell wall. The subtilin leader is preferably attached through its N-terminal end to the spacer, therefore it is not subject to cleavage by the signal peptidase. On a wild-type subtilin preprotein, the leader sequence is normally cleaved at its C-terminal end, thus generating the mature subtilin protein.

[0041] The complete subtilin leader segment is 24 residues long **(8)**, and this together with a 20-residue spacer places 44 residues at the C-terminus of the lantibiotic peptide. In a preferred embodiment, the chimeric sublancin Lantibody Display Peptide comprises the formula:

$$\textit{sunA}\text{-}PG_{20}\text{-}S_{L1\text{-}24}$$

[0042] In a preferred embodiment, a sublancin-derived Lantibody Display Peptide is generated by transfecting a B. subtilis 168 *ermΔSun* host cell with a linearized mutagenesis plasmid pAV2 containing the sequence of *sunA-PG$_{20}$-S$_L$*. The pAV2 vector is depicted in Figure 7. The transformed host is then cultured in medium to allow the Lantibody Display Peptide to be expressed.

[0043] Preferably, the bacterial host transformed with the inventive mutagenesis vector is a sublancin-producing host. More preferably, the sublancin producing host is a strain of Bacillus subtilis such as B. subtilis 168. Most preferably, the host is B. subtilis LPeΔ*sun*A.

[0044] When the gene for this peptide is expressed in *B. subtilis* 168, the expected sequence of events is as follows. The gene is transcribed, translated and posttranscriptionally-modified to give the precursor peptide in Figure 2. The precursor peptide is then secreted across the cytoplasmic membrane by means of sublancin leader segment which is recognized by the normal sublancin transporter system. Once in the cell wall, the sublancin leader is cleaved in the usual manner. Whereas sublancin itself would normally diffuse toward the surface and be released, the presence of the subtilin leader segment at the C-terminal causes the entire molecule to be retained within or on the cell wall.

[0045] Any suitable growth media can be used to culture the lantibody expressing cells, e.g., media comprising nitrogen sources such as yeast extracts, soy tripticase, peptone, salts, metal ions, citric acid, buffers, carbohydrates such as glucose, glycerol, lactose, sucrose, molasses, chalk, phosphates, ammonium sulfate and oil.

[0046] The variable region of a wild-type lantibiotic gene, or preferably a sublancin gene, can be mutagenized by any art-recognized methods and subcloned into the mutagenesis vector for transfection and stable integration through homologous recombination of the mutagenized gene into the chromosome of a susceptible host cell. A sublancin mutant that can be expressed and secreted by B. subtilis 168 is an object of this invention. The production of a mutated, mature sublancin protein is demonstrated hereunder.

[0047] Strategies are readily available to collect lantibody-expressing cells in order to obtain and characterize the lantibody that is responsible for binding to any given target molecule. Lantibody expressing cells which bind to a target molecule can be detected and purified by reacting the cells with an anti-sublancin antibody or an anti-target antibody followed by passage of the cells over any recognized methods for separating and enriching viable cells such as an immunoadsorption column, magnetic bead separation or flow cytometry. Following purification, the enriched cells are eluted and collected for analysis. The structure of the lantibody can be determined by sequencing the protein or polypeptide, or the gene that encodes it.

[0048] The lantibody protein or polypeptide or a mutant thereof, can be sequenced as follows: the proteins are reduced and alkylated in preparation for protease digestion. 75 μl 50 mM dithiothreitol (DTT) and 150 μl 100 mM iodoacetamide, both in 0.2 Na M borate, pH 8.0, are added to 100 μg lyophilized peptide and incubated overnight at room temperature in the dark. 1 ml 0.1% acetic acid, 0.01% trifluoroacetic acid (TFA) are added and the mixture immediately purified by HPLC as previously described **(4),** and fractions collected. The fractions containing alkylated peptide, as determined by subjecting 0.5 μl of each to matrix assisted laser desorption/ionization-time of flight MS (MALDI-TOF MS), are lyophilized and resuspended in 100 μl 100 mM NH_4CO_3, 1 mM $CaCl_2$, pH 8.0. Sequencing-grade trypsin (Sigma, St. Louis, Mo) is added at a 1:50 enzyme to substrate ratio and the mixture incubated at 37^0C for 4 hours. 1 ml of 0.1% acetic aced, 0.01% trifluoroacetic acid (TFA) is added and the mixture immediately purified by HPLC as above, except the first step of the elution profile is 15% B over 5 minutes and the second step is from 15 to 65% B over 20 minutes. The fractions containing peptide fragments, as determined by subjecting 0.5 μl of each to MALDI-TOF MS, are lyophilized and resuspended in 25 μl 0.1% acetic acid, 0.01% TFA and sequenced using nanoESI-MS/MS.

[0049] The gene encoding the lantibody can be sequenced by any variety of art-recognized methods **(6)**.

[0050] A population of surface-displayed lantibodies can be challenged by a biologically relevant target molecule of interest, which is preferably a nucleophilic group within a

polypeptide sequence. Cells which bind to the target are those which synthesize a Lantibody having the ability to bind to the target.

[0051] Nucleophilic target molecules that bind to the lantibody include but are not limited to nucleophilic groups located on antigens, virus particles, bacterial cells, more preferably gram-positive bacterial cells, bacterial spores, vegetative bacterial cells, and the proteins and polypeptides on any of the aforementioned biologically relevant molecules including enzymes and receptors. Preferably, the target molecule has a nucleophilic group within a polypeptide chain. A nucleophilic group can also be located on a polypeptide surface where the polypeptide is in the form of a tertiary or quarternary complex.

[0052] An antigen being attached to a lantibody would be partially or completely blocked from interacting with its cognate binding partner. A binding partner may be an antibody or a receptor in either soluble or membrane-associated form.

[0053] Bacterial cells including but not limited to *Bacillus cereus T, Bacillus megaterium, Bacillus subtilis, Staphylococcus aureus* or *Streptococcus pyogenes*, would be killed or growth inhibited by an attached lantibody.

[0054] In accordance with the present invention, a lantibody attached to a spore would prevent spore outgrowth.

[0055] A virus particle that had its receptor binding sites blocked by a lantibody would preferably be rendered permanently non-infectious.

[0056] An enzyme that had its catalytic site blocked by an attached lantibody would preferably be completely or partially inactivated. Alternatively, a lantibody may become attached to a regulatory site on an enzyme, whereupon its activity would be partially or permanently modulated according to the function of the regulatory site. Enzymes include but are not limited to proteases and protein kinases.

[0057] The display peptide was designed with the intention of allowing a functional lantibody to be displayed on the outer surface of the cell wall. This object of the invention has been achieved by a series experiments some of which are described by way of the following non-limiting examples.

Example 1

Construction of a mutagenesis system to create mutations in the sublancin gene, and introduction of the mutations into the chromosome.

[0058] The strategy that was developed for sublancin mutagenesis is the subject of a previous provisional patent application Ser. No. 60/215,449. It is similar to the strategy used for subtilin mutagenesis **(9)** which was to construct a host-vector pair with the vector being a plasmid used for the construction and propagation of the mutant gene, and the host being a *B. subtilis 168* mutant with a deletion in the sublancin gene and an *erm* resistance marker. The plasmid vector was designed to contain homologies to the regions flanking the sublancin gene together with a *cat* selective marker, such that a double-recombination between the plasmid and the host results in replacement of the *erm* gene in the host with the mutagenized sublancin gene, together with the *cat* marker. After this recombination, the mutagenized sublancin gene is located precisely where the native gene had been, so that effects on expression would be minimized. Appropriate recombinants are identified by their loss of erythromycin resistance, and gain of chloramphenicol resistance.

[0059] Bacterial strains, cloning vectors, and culture conditions. Bacterial strains and cloning vectors are listed in Table I. Sublancin and its analogs were expressed and isolated as previously described **(4)**. Competent *B. subtilis* 168 cells were prepared according to Young and Wilson **(10)**.

[0060] Table I

Bacterial Strains		
Strain	Description	Source
BR151	Wild type *Bacillus subtilis* 168	BGSC[a]
B. subtilis LPeΔ*sunA*	*Bacillus. subtilis* 168 in which the sublancin gene contains an in-frame deletion, and an *erm* selective marker	Invention
B. subtilis sunA'	Sublancin producer strain made by transforming pLPVc into *B. subtilis* LPeΔ*sunA*	Invention
JM109	Standard *E. coli* cloning strain	Life Tech.[b]
JM101	Standard *E. coli* cloning strain	Life Tech.[b]
B. cereus T	Strain used to assay sublancin	BGSC
Cloning Vectors		

Plasmid	Description	
pSUB8kb	An 8kb fragment from *Bacillus subtilis* 168 containing the sublancin gene in pTZ18R	Ref[c]
pTZ18R	Standard cloning vector	USB[d]
pLPe□*sunA*	Plasmid used to construct *B. subtilis* LPe∆*sunA*	Invention
PLPc	Plasmid used to integrate a sublancin gene into the chromsome of *B. subtilis* LPe∆*sunA*	Invention

Bacterial strains and plasmids used in this work. [a]Bacillus Genetics Stock Center, University of Ohio, Columbus OH. [b]Life Technologies, Gaitherburg MD. [c]**(4)**. [d]United States Biochemical Corp., Cleveland OH.

[0061] Cloning by PCR. The polymerase chain reaction (PCR) was used to generate the DNA fragments used for the construction of the plasmid vectors used for mutagenesis. Plasmid pSUB8kb was used as the template DNA for the PCR reactions. The sequences of the oligonucleotides used as primers in the PCR reactions are listed in Table II, and the template regions corresponding to these oligonucleotides are identified in Figure 3. The reactions were performed using *pfu* DNA polymerase (Strategene, La Jolla, CA) during 30 cycles of denaturation at 95°C for 30 sec, annealing at 50°C for 90 sec, and extending at 72°C for 3 min. The PCR fragments were cleaved with EcoRI and HindIII and cloned into the EcoRI-HindIII site of pTZ, which was propagated in *E. coli* JM101 or JM109. The PCR primers were designed in such a way that the plasmid constructs could be assembled by a sequence of ligation and cloning steps that added one PCR segment at a time, with each addition being confirmed by cloning and restriction analysis before adding the next segment. After the assembly was complete, the entire insert was subjected to dideoxy sequence analysis to confirm that it had been correctly assembled and that it contained no unintended mutations.

[0062] Table II

	Oligonucleotide Sequence written 5' to 3'
LPHF1 SEQ ID No. 3	GACTGAATTCCGGCTCTAAAGCGATTC EcoRI
LPHR1 SEQ ID No. 4	GGACTAAGCTT GGATCCGAATTGGTTGTAATACAC HindIII BamHI
LPHF2 SEQ ID No. 5	GCAACGAATTC GGATCCGTGTATTACAACCAATTC EcoRI BamHI
LPHR2 SEQ ID No. 6	TCGAAAAGCTT GTTAACCTTTTCCATTTGTAAAACC HindIII HincII

LPHF3 SEQ ID No. 7	TGGCAGAATTC GTTAACTATCGTCAATTCTGC EcoRI HincII
LPHR3 SEQ ID No. 8	GGAGCAAGCTT CAGCAAGACCCACAACG HindIII
LPVF2 SEQ ID No. 9	Same as LPHF2
LPVR2 SEQ ID No. 10	GGATGAAGCTT CTCGAGTTTAACTTCTTTA HindIII XhoI
NLPVF3 SEQ ID No. 11	GTAGGAATTC CTCGAGGAACTCGAAAACC EcoRI XhoI
LPPMR2 SEQ ID No. 12	GGAGCAAGCTTTTATCTGCAGAATTGACGATAG HindIII PstI
LPVF4 SEQ ID No. 13	GATTGAATTCGGCGCCGTTGCTTGTCAAAAC EcoRI
LPVR4 SEQ ID No. 14	Same as LPHR3
L13 SEQ ID No. 15	GTGTATTACAACCAATTCTG
L15 SEQ ID No. 16	TTGTGGCTACAATGTGCTAG

Sequences of Oligonucleotides used for PCR and sequencing primers and hybridization probes. The locations of the template regions corresponding to the primers are shown in Figure 3. LPV oligos were used to construct the pLPc mutagenesis vector, and the LPH oligos were used to construct the pLPeΔ*sunA* plasmid, which was used to construct *B. subtilis* LPeΔ*sunA*. LPVF and LPHF oligos prime in the forward direction, and the LPVR and LPHR oligos prime in the reverse direction. Oligonucleotide L13 was used as a sequencing primer that was about 220 nt upstream of the sublancin gene, and L15 was used as a hybridization probe within the sublancin gene.

A. Construction of pLPVc vector by PCR cloning.

[0063] The primary vector, pLPVc, was constructed from components synthesized by PCR and assembled in the EcoRI-HindIII site of the *E. coli* plasmid pTZ. The complete assembled EcoRI-HindIII insert sequence of pLPVc is shown in Figure 4. This insert contains a 650 base pair upstream chromosomal homology, followed by a *cat* gene that has been inserted into an engineered BamHI site, followed by the presublancin (*sunA*) gene, which contains a translationally-silent XhoI site in the leader region of SunA, and the natural PstI site in the C-terminal region, which is followed by a 650 by of downstream chromosomal homology. This plasmid constitues a cassette-mutagenesis system, in which the sequence of the mature region of SunA can be modified by replacing the XhoI-PstI fragment with a mutagenized sequence.

B. Construction of the *B. subtilis* LPeΔ*sunA* host.

[0064] The pLPVc plasmid was then modified in order to construct pLPHe, which was used to engineer a deletion in the chromosomal *sunA* gene and replace the cat gene with an *erm* gene. The pLPHe plasmid, shown in Figure 5, contains an *erm* gene in the BamHI site, and 47 codons are removed from the central region of the 56-codon *sunA* ORF. The remaining 9 codons are in-frame in order to minimize any effects of the deletion on the expression of downstream genes that may be required for sublancin biosynthesis. This in-frame construction was to permit this host to be used for expression of sublancin genes in *trans*, from a plasmid, as well as by integration into the chromosome.

[0065] The use of these plasmids in making sublancin mutants is diagrammed in Figure 4. First, a double-recombination between pLPHe and the *B.* subtilis 168 chromosome replaces the *sunA* gene with an *erm* gene. The resulting *B.* subtilis LPeΔ*sunA* is erythromycin resistant and does not produce sublancin. The pLPVc plasmid was then used to introduce a mutagenized copy of *sunA,* at precisely the same location occupied by the original *sunA* gene by means of a double-recombination that replaces the *erm* gene and *sunA* deletion with a cat gene and the mutant *sunA'* gene. The cat gene is placed upstream from the *sunA'* promoter so as to not interfere with expression of the *sunA'* gene.

[0066] A halo assay was used to compare the amount of antibiotic production by B. subtilis 168 with that of the LPeΔ*sunA* deletion strain. The amount of antibiotic activity produced by a bacterial colony was determined by its ability to inhibit outgrowth of *Bacillus cereus* T spores to produce a halo around the colony. *B. cereus* T spores were prepared by suspending 250 mg of lyophilized spores **(11)**, in 30 ml of sterile water and subjecting them to heat shock for 2 hr at 65 ^{0}C. The spores were centrifuged and resuspended in 50 ml of 10% ethanol. This solution was used to spray Medium A plates on which colonies had grown to a diameter of 1 mm. The plates were incubated 5 to 12 hr to allow the spores to germinate and outgrow. The diameters of the clear halos were used to compare the amount of antibiotic produced by the colonies.

[0067] As shown in Figure 6, the difference is dramatic, with the wild-type strain giving a large halo, and the deletion strain a barely detectable one. Under these growth conditions,

sublancin constitutes a large majority of the antimicrobial activity produced by *B.* subtilis 168.

C. Integration of the *sunA'* gene restores sublancin biosynthesis.

[0068] The sublancin gene subcloned into the plasmid pLPVc is *sunA'*, which is identical to *sunA* except for the translationally-silent mutations used to create the XhoI site. Since *sunA'* encodes the same amino acid sequence as the natural *sunA* gene, placing *sunA'* into the chromosome at the location originally occupied by *sunA* would be expected to restore sublancin production. Figure 6 shows a halo assay demonstrating that recombinant cells having the *sunA'* gene integrated into the chromosome are restored in their expression of antimicrobial activity.

[0069] The molecular mass of sublancin was determined using electrospray ionization mass spectroscopy (ESI-MS) on a single quadropole ion-trap mass spectrometer in positive ion mode (LCQ, Finnigan, San Jose, CA). The source conditions were as follows: sheath gas flow, 40 units, ESI spray voltage, 5 kV, capillary temperature 200°C, capillary voltage 46 V. MS data were acquired on a Windows NT worksation running the LCQExplore software package (Finnigan). MALDI-TOF MS were carried out in positive-ion mode (Proflex, Bruker, Manning Park, Ma). Sinapinic acid, dissolved in acetonitrile, 0.1% TFA (3:7), was used as matrix. The sample and matrix were applied to the sample target (Bruker) according to the sandwich method of Kussman *et al.* **(12)**. Tryptic digest fragments were sequenced using tandem MS/MS, using the nanospray adapter on the Finnigan LCQ (nanoESI-MS/MS). Nanospray capillaries(Protana, Odense, Denmark) were used to supply the sample to the LCQ at a very low flow rate (1-10 nl/min). The source conditions were as follows: ESI spray voltage, 0.8-0.1 kV, capillary temperature 200°C, capillary voltage 41 V, MS/MS relative collision energy, 80%. Sequence interpretation was assisted by the use of AminoCalc software (Protana).

[0070] The active peak emerged from the HPLC column at the same gradient position as natural sublancin (data not shown), and mass spectral analysis using MALDI-TOF gave a major species with a molecular mass of 3881 Da. The molecular weight species

corresponded to the 3881 Da positive control using natural sublancin. This molecular weight is also very close to the 3878 molecular weight previously reported for sublancin **(6)**. These results demonstrate that *B. subtilis* LPeΔ*sunA* host has been stably converted to express sublancin by the pLPVc plasmid, and that the presence of the cat gene upstream from the *sunA* gene does not interfere with *sunA* expression.

Example 2

Construction and expression of a soluble sublancin structural mutant.

[0071] The pLPVc plasmid was tested for its utility in the construction and expression of mutant sublancin peptides. One mutant was Dha16T, in which the Dha residue was replaced with a threonine. The ability to generate the Dha16T mutant addressed the question as to whether the sublancin processing machinery is sufficiently flexible in its recognition and processing of the presublancin peptide to convert a threonine residue at position 16, which is normally a serine, to the corresponding Dhb residue. The success in obtaining the mutant demonstrates that the sublancin processing machinery is relatively tolerant of structural changes in its substrate. These results are strongly indicative of the ability of sublancin biosynthesis pathway to process precursors with a variety of other changes. These positive results also provide the basis on which to conduct a comprehensive structure-function analysis of sublancin.

[0072] This plasmid construct was also tested for its biological activity toward inhibition of bacterial outgrowth, and found to be active. Mass spectral analysis showed that the molecular weight of the Dha16T mutant is exactly as expected for the threonine having undergone dehydration. This demonstrates that the threonine had been correctly processed to a Dhb residue, and that the sublancin processing machinery is capable of correctly recognizing and processing a residue that is not normally a component of the sublancin molecule.

Example 3

Construction of the Display Peptide Consisting of the sublancin prepeptide with a 20-residue polyglycine and the subtilin leader segment at the C-terminus of the prepeptide.

[0073] Figure 2 shows the basic design of the display peptide. The actual sequence that was constructed is shown in Figure 7. As outlined in Figure 2, this peptide consists of the sublancin precursor peptide (which contains the sublancin leader and the sublancin mature segment), fused at its C-terminus to a glycine residue spacer, followed by the subtilin leader segment. This sequence was constructed in the pLPcat vector shown in Figure 5, and transformed into the chromosome of the *B.* subtilis 168 *ermΔSun* host, as shown in Figure 5.

Example 4

Expression of the Lantibody Display Peptide.

[0074] The transformed host was cultured in Medium A to allow the Lantibody Display Peptide to be expressed. Expression was monitored by observing the appearance of the Display Peptide in the cytoplasm of the cell, the membrane, the cell wall and in the extracellular medium. The peptide was detected using polyclonal antibodies raised in rabbits against a sublancin-KLH conjugate as an antigen. Solubilized components from the different cell fractions were applied to a reversed-phase HPLC column **(4)**, and the fractions were analyzed using mass spectroscopy. One of the most important observations was that the cell wall contained considerable amounts of a species with a molecular weight corresponding to the full-length peptide as shown in Figure 8. Moreover, none of this full-length peptide appeared in the extracellular fluid, showing that it is tenaciously bound to the cell wall as was predicted from the ability of the subtilin leader segment to bind to the cell wall **(13)**. However, several degradation products of the full-length peptide did appear. Several extracellular components corresponded to products resulting from cleavage within the polyglycine spacer, and several others which corresponded to products resulting from cleavage within the subtilin leader segment. Of particular interest

was an extracellular species with a molecular weight corresponding to amino acid residues 1-77, which would result if 4 residues were cleaved from the C-terminal end. The fact that this peptide was released into the culture supernatant whereas the full length (1-81) peptide was not, shows that the tetrapeptide sequence at the C-terminal end is crucial to provide the tight binding to the cell wall. Those peptides released into the medium had antimicrobial activity, showing that the C-terminal modification of sublancin does not disrupt the posttranslational modification process. Those results also demonstrate the ability of the sublancin Display Peptide to recognize and bind to a target substrate, i.e., the polyclonal antibody, and that this activity is not lost as a result of the structural modifications to the protein.

Example 5

The location of the Lantibody Display Peptide in the cell wall is near the surface.

[0075] In order to fulfill the concept of the Lantibody Display Library, it is necessary that the Lantibody Display Peptide be located near the surface of the cell where it can interact with ligands so that the screening procedures, as outlined above, can be carried out. To explore this hypothesis, cells expressing the Lantibody Display Peptide as shown in Figure 8 were centrifuged out of the culture medium and resuspended in buffer. The cell suspension was treated with anti-sublancin antibodies, and washed to remove any unbound antibody. To determine whether anti-sublancin antibodies were bound to the surface of the cells, goat anti-rabbit antibodies that were conjugated with horseradish peroxidase were added and allowed to adsorb to any antibodies on the surface of the cells. The cells were washed and the peroxidase color reagent was added. The cells quickly became intensely blue, showing that rabbit antibodies were present. Control cells that lacked the sublancin gene were colorless, showing that the color was indeed because of the presence of sublancin within the cell-wall matrix. These results demonstrate that the location of the sublancin within the cell wall is accessible to antibodies that have diffused into the matrix. Under the transfection conditions, the Sublancin Display Peptide is the primary species of protein in the cell wall fraction, so the Sublancin Display Peptide is

responsible for the binding to sublancin antibodies.

[0076] To determine whether the sublancin is embedded deeply within the cell-wall matrix or near or on the surface, experiments were conducted using magnetic beads that were coated with anti-rabbit antibodies (beads obtained from Dynal, Inc.). These beads were added to a suspension of Sublancin Display Peptide-producing cells that had been treated with anti-sublancin antibodies, and thoroughly washed. After incubating for 16 hr, microscopic examination showed that the cells had aggregated onto the surface of the beads, indicating that the cells were coated with rabbit antibodies that could interact with the anti-rabbit antibodies on the beads. Cells that did not contain a gene for sublancin did not show such aggregations, which establishes that the interaction between cells and beads is a result of the presence of the Sublancin Display Peptide very near to or on the surface of the cells.

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